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Anomalous electrophoretic migration of newly synthesized ribosomal RNAs and their precursors from cells with *DKC1* mutations

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ABSTRACT

Mutations in the X-linked gene, *DKC1*, encoding dyskerin, cause dyskeratosis congenita by leading to decreased telomerase activity and causing short telomeres. Dyskerin is also a pseudouridine synthase that modifies nascent ribosomal and other RNAs and it is not known if this function is affected by the mutations. Here we show that newly synthesized ribosomal RNA, extracted from human and mouse cells with pathogenic mutations, shows anomalous mobility in agarose gels under certain denaturation conditions. The anomalously migrating RNA is turned over rapidly. Analysis of ribosomal RNA in these cells suggests the altered mobility is due to inefficient pseudouridylation.

1. Introduction

Pseudouridine (Ψ) is present in RNAs throughout all kingdoms of life but the role of this modified nucleoside is not fully understood [1]. Conversion of specific uridines (U) to Ψ takes place after transcription and is accomplished by enzymes called Ψ synthases. In bacteria these are protein only enzymes that select specific Us and catalyse their isomerization [2]. Similar enzymes exist in eukaryotes where they catalyse Ψ formation in tRNAs and possibly 5S rRNA but in eukaryotic rRNA pseudouridylation is carried out by a complex of four proteins and one guide RNA called an H/ACA box small nucleolar ribonuclear protein (snoRNP) [3]. The RNA, an H/ACA box snoRNA, guides the complex to a specific U residue and one of the proteins, Cbf5 in yeast, NAP57 or dyskerin in vertebrates, is the Ψ synthase. Since a large number of different snoRNAs are present this system can select and pseudouridylate a large number of different Us in 5.8S, 18S and 28S ribosomal RNAs and in the snRNAs that mediate mRNA splicing.

In vertebrates the 4 H/ACA box snoRNP proteins are also found in association with telomerase RNA [4,5], telomerase reverse transcriptase and other proteins in the telomerase complex, responsible for synthesizing the telomeric DNA repeats.

In humans mutations in genes encoding five components of the telomerase complex and one component of telomeres, cause the bone marrow failure syndrome, dyskeratosis congenita (DC), providing strong evidence that DC arises due to defective telomere maintenance [6]. The major X-linked form of DC is caused by mutations, mainly missense, in the *DKC1* gene encoding dyskerin. An ongoing debate concerns whether defective ribosome biogenesis contributes to the phenotype or whether telomerase defects alone can account for the pathology in these patients. Since X-linked DC is more severe than forms of the disease arising from telomerase mutations one could argue that the extra severity is due to ribosome biogenesis problems, though a counter argument could be that in X-linked DC the telomerase activity is more severely affected than in the other forms, in which there is haploinsufficiency for telomerase RNA or the telomerase reverse transcriptase. Examination of cell lines from DC patients shows no evidence for defects in ribosome biogenesis or alterations in Ψ levels [7], though only a small number of different mutations have been examined in this way. Necessarily these have been milder mutations, since more severe mutations inhibit proliferation, making extensive cell culture

Abbreviations: U, uridine; Ψ, pseudouridine; snoRNP, small nucleolar ribonuclear protein; ES cell, embryonic stem cell; MEF, mouse embryo fibroblast

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problematic. On the other hand mice, or mouse embryonic stem cell (ES cells), containing pathogenic *Dkc1* mutations, show slightly decreased Ψ levels and delayed kinetics of rRNA synthesis, and alterations in snoRNA levels [8,9].

The foregoing discussion boils down to two questions. Do pathogenic *DKC1* mutations affect pseudouridylation? If so do the changes in pseudouridylation affect the outcome of DC. In this paper we are able to contribute to the first of these questions. We made a serendipitous observation that rRNA molecules from cells with *DKC1* mutations have altered mobility in formaldehyde agarose gels, demonstrating a biophysical difference from RNA from wild-type cells. We show that this mobility difference is seen in both human and mouse cells, is most pronounced in newly synthesized RNA and correlates with a detectable difference in the level of pseudouridylation.

2. Materials and methods

2.1. Cell lines

The generation and culture of WT, $Dkc1^{\Delta 15}$ and $Dkc1^{A353V}$ ES cells, and WT and $Dkc1^{\Delta 15}$ mouse embryo fibroblasts (MEF) cells has been described previously [8,9]. MEF cells were maintained at 37 °C in a humidified atmosphere of 3% O_2/5% CO_2.

Human fibroblast cell line (GM01774, Coriell, USA) is from a dyskeratosis congenita male proband who is a hemizygous for an in frame 3 bp deletion of nucleotides 201–203 of the *DKC1* gene resulting in the deletion of leucine at position 37 (ΔL37). The fibroblast cell line (GM01787) is from the grandmother of the proband, she is a possible carrier had no clinical symptoms. These fibroblast cells were cultured in DMEM supplemented with 15% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin and maintained at 37 °C in a humidified atmosphere of 21% O_2/5% CO_2.

2.2. Pulse-chase labeling of RNA

MEF and ES cells were preincubated for 45 min in methionine-free medium and then incubated for 30 min in medium containing L-[methyl-^{3}H]methionine (50 μCi/ml). The cells were then chased in non-radioactive fresh medium for various times.

2.3. Agarose gel electrophoresis

Total RNA was extracted from ES cells and MEF cells by using TRIzol Reagent (Invitrogen, Carlsbad, CA). RNA was mixed with 2X volumes RNA Sample Loading Buffer (Deionized formamide 62.5% (v/v), formaldehyde 1.14 M, bromphenol blue 200 μg/ml, xylene cyanol 200 μg/ml, MOPS-EDTA-sodium acetate at 1.25× working concentration.) and denatured for 1, 5 or 20 min at 65 °C. Total RNA was separated on 1.25% agarose formaldehyde (2.2 M) gel using 1× MOPS electrophoresis running buffer (0.02 M MOPS, 0.005 M Sodium Acetate, 0.001 M EDTA and 0.001 M EGTA). After 6 h electrophoresis using 100 V voltage, RNA was transferred onto nylon membrane (GE Healthcare). The membranes were sprayed with EN^{3}HANCE Spray (Perkin–Elmer) and exposed to X-ray films at −80 °C.

2.4. Detection of Ψ using the ligation method

We used the method previously described [10,11]. The oligonucleotides were

N1 5′-ACT CCC GCC GTT TAC C-3′
D1 5′-N6-phenanthren-9-yl-ACT CCC GCC GTT TAC C-3′
N2/D2 5′-CTA CCT TAA GAG AGT CAT AGT T-3′

RNA was synthesized in vitro by incorporating the T7 promoter sequence into an oligonucleotide and amplifying a 150 bp sequence containing the target sequence and then using T7 RNA polymerase from NEB (Ipswich, MA) according to the manufacturer's recommendations. Annealing reactions (25 μl) contained 3.75 pmol of in vitro transcribed RNAs or 10 μg mouse RNAs as well as 13 pmol ^{32}P-labeled N1 or D1 and 9.4 pmol N2/D2. After annealing by cooling from 95 °C to 4 °C for 45′ ligation was carried out at 27 °C for 15′. Loading volumes were adjusted to give equal intensity with the N-oligonucleotide reactions and the same volumes from the D reactions were loaded.

3. Results

3.1. Altered mobility of ribosomal RNAs from cells with mutant dyskerin

In our studies on the effect of dyskerin mutations on ribosome biogenesis we use the technique of pulse-chase labeling of RNA using ^{3}H methyl-methionine as the labeled reagent. RNA is rapidly labeled, by post-transcriptional addition of labeled methyl groups, after addition of ^{3}H methyl-methionine and the specific activity of the methionine pool falls rapidly when cold methionine is added. Fig. 1A shows that in ES cells with the $Dkc1^{\Delta 15}$ mutation [9] this technique reveals a slightly delayed appearance of mature 28S rRNA in the mutant cells and slower processing of the 32S precursor RNA during the chase [8,9]. In some of our experiments the mobility of mature and precursor RNAs from mutant cells was higher than that from wild-type cells. Testing different experimental conditions led us to conclude that there is a consistent increase in the mobility of newly labeled RNA in cells with a *Dkc1* mutation when shorter than recommended times are used for denaturation in 2.2 M formaldehyde and 50% formamide at 65 °C. Fig. 1 shows that with 1′ denaturation mature and precursor ribosomal RNAs from mutant cells migrate faster than the corresponding molecules from wild-type cells. Moreover, the same phenomenon is seen with different mutants and in different species. Thus the faster mobility of RNAs from mutant cells is also seen with RNA from $Dkc1^{\Delta 15}$ MEF cells (Fig. 2), from $Dkc1^{A353V}$ ES cells and interestingly from $DKC1^{\Delta L37}$ human fibroblasts [4,12] (Fig. 3).

In order to understand the basis of the mobility differences between rRNA species from normal and *Dkc1* mutant cells we performed a pulse chase experiment using normal and $Dkc1^{A353V}$ male ES cells generated by gene targeting [8]. RNA was extracted with no cold methionine chase or after a 15 min chase and RNAs were denatured for 1′, 5′ or 20′ and run on the same formaldehyde containing agarose gel. The results show that in this experiment the faster mobility of the mutant RNAs is still evident after 20′ denaturation and that all RNAs run slower with longer denaturation time (Fig. 3). These results suggest that denaturation of ribosomal RNA is a time dependent process under the conditions we use and that RNA from normal cells is more easily denatured than that from mutant cells.

3.2. Higher mobility rRNA is more rapidly turned over

We observed that when using ^{3}H–U as the source of label much less difference was seen in mobility between RNA from mutant cells and RNA from wild-type cells (data not shown). We reasoned that the major difference between U and methyl-methionine as the source of the label is that U pools are more slowly turned over than methionine pools so RNA labeled with U consists largely of steady-state rather than rapidly turned over RNA, leading us to consider that the higher mobility RNA seen in the mutant cells was turned over faster than RNA of normal mobility. To test this idea we re-

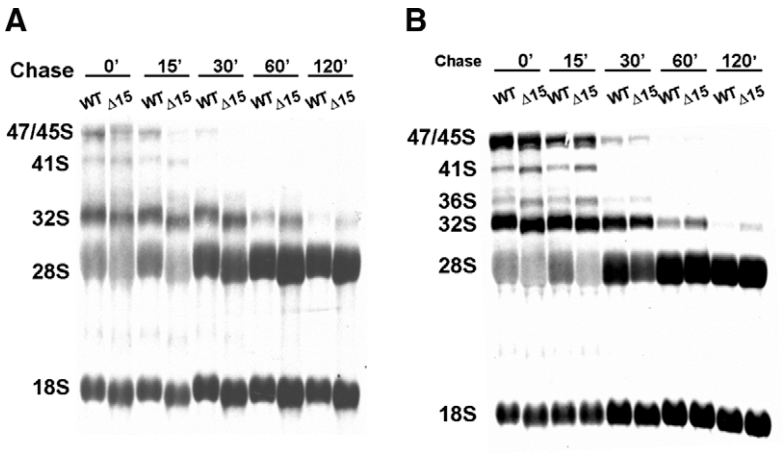

Fig. 1. Pulse-chase analysis of RNA from WT and *Dkc1*$^{\Delta 15}$ (Δ15) ES cells. Cells were labeled with ^{3}H-methyl-methionine for 30′and chased with cold methionine for the times indicated. Samples were denatured for 5′ (A) or 20′ (B).

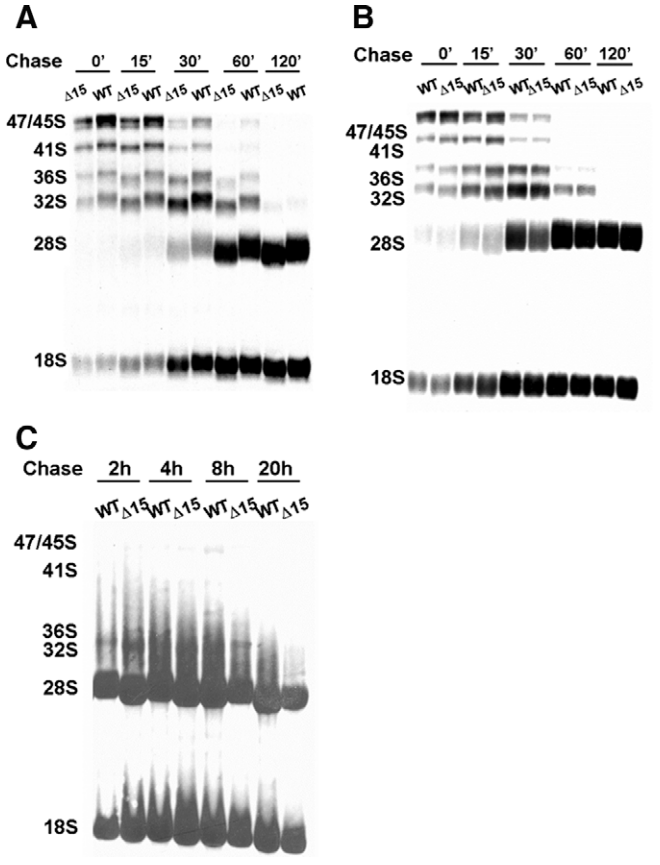

Fig. 2. Pulse-chase analysis of RNA from WT and *Dkc1*$^{\Delta 15}$ (Δ15) MEF cells. Cells were labeled with ^{3}H-methyl-methionine for 30′ and chased with cold methionine for the times indicated. Samples were denatured for 5′ (A and C) or 20′ (B).

peated the pulse chase experiment but with longer periods of chase with cold methionine. The results show that some mobility difference in the mature 18S and 28S RNAs is still present after a 2 h chase but this is decreased at 4 h and is virtually absent at 8 h and 20 h. Any apparent mobility differences at this stage, affecting the leading edge of the bands is presumably due to loading differences. These results suggest that the mutant RNA with a higher mobility, presumably due to differences in pseudouridylation, has a shorter half-life than the RNA with normal mobility.

3.3. Decreased pseudouridylation at a specific site in 28S RNA from mouse cells with mutant dyskerin, measured by a ligation assay

The simplest way of explaining the finding of a fraction of high mobility rRNA from cells with a mutated dyskerin is that the RNA is incompletely pseudouridylated compared with RNA from wild-type cells and that the pseudouridylation differences account for the differences in mobility. In support of this idea is our previous finding comparing wild-type and *Dkc1* mutant male ES cells, where we found after labeling with ^{32}P-orthophosphate for 3 h that significantly less Ψ was present in 18S and 28S rRNA from *Dkc1*^{A353V} or *Dkc1*^{G402E} ES cells than in wild-type cells [8]. To acquire supporting evidence for this interpretation we decided to assess the pseudouridylation status of RNA from *Dkc1* mutant cells by a different and independent method. We chose to use a method based on the discovery that the presence of a Ψ in RNA can alter the efficiency with which two oligonucleotides annealed to the RNA can be ligated, when the ligatable ends of the oligonucleotides abut the Ψ [10]. The difference in ligation efficiency is enhanced when the 5′ residue to be ligated is N6-phenanthren-9-yl-A. After preliminary trials with several Ψ sites we found the discrimination to be optimum with the Ψ at U3414 in 28S RNA. This U is the mouse equivalent of human residue 28S U 3727. The mouse snoRNA that guides the snoRNP complex to this residue is MBI-1 [13]. Fig. 4 shows that when equivalent molar amounts of RNA prepared in vitro and RNA from mouse tissues are annealed with non-discriminating oligonucleotides the amount of ligation product is equal whereas using the discriminating oligonucleotides there is much less ligation product when using mouse RNA as a template. Then comparing RNA from mutant and wild-type mouse cells we see that there is consistently more ligation product when mutant RNAs are used as the template. The amount of ligation product, however does not approach the amount with the in vitro RNA, indicating that a small fraction of the molecules have a U at position 3414 while most have a Ψ. This semi-quantitative experiment demonstrates a clear and consistent difference in the level of pseudouridylation at residue 3414 between RNA from wild-type cells and RNA from *Dkc1*^{A353V} and *Dkc1*$^{\Delta 15}$ ES cells.

4. Discussion

We have found that ribosomal RNAs from cells with a mutated *DKC1* gene consistently have a higher mobility in denaturing agarose gels under certain denaturation conditions. Since dyskerin is a Ψ synthase that catalyses the conversion of U to Ψ in ribosomal

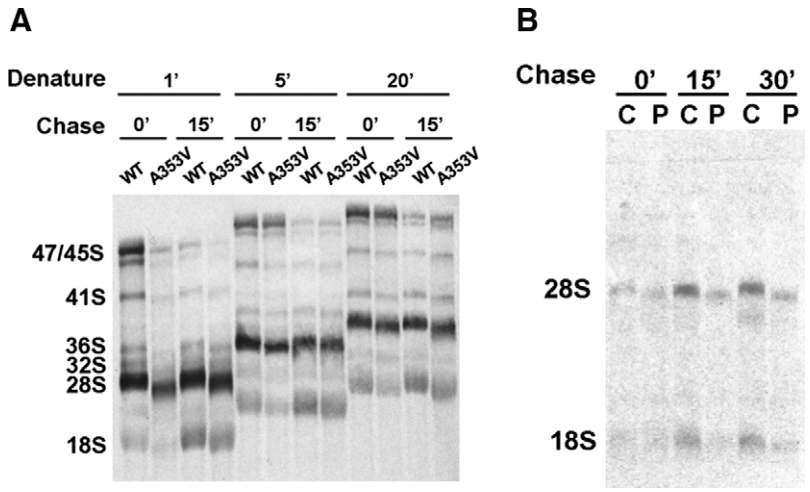

Fig. 3. Pulse-chase analysis of RNA from WT and *Dkc1*^{A353V} ES cells and human fibroblast cell lines. (A) Cells were labeled with ^{3}H-methyl-methionine and chased with cold methionine for 15′. Samples were denatured for the indicated times. (B) Cells were labeled with ^{3}H-methyl-methionine for 15′ and 30′. C, control (GM01787). P, X-linked DC patient (GM01774).

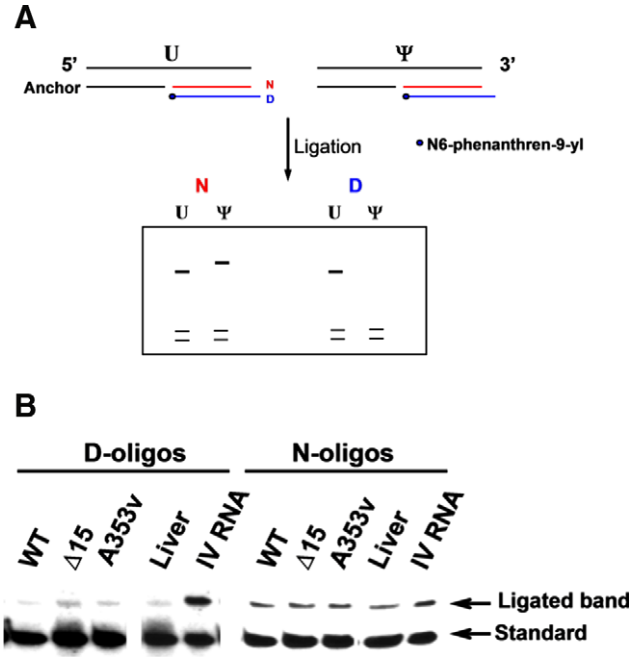

Fig. 4. Analysis of pseudouridylation at U 3414 in mouse 28S RNA. (A) Ligation with modified D oligonucleotides is less efficient with a Y in the template. (B) Incomplete pseudouridylation of residue U3414 in Δ15 and A353V ES cells compared with WT. In the experiment shown mouse liver RNA and in vitro synthesized template were loaded to equalize the signals with the N oligonucleotides. Then WT, Δ15 and A353V RNAs were also loaded in amounts that gave equal signals with the N oligonucleotides.

RNAs this difference is likely to be due to differences in pseudouridylation. This interpretation is consistent with our previous results showing decreased Ψ levels in newly synthesized rRNA from ES cells containing *Dkc1* mutations A353V and G402E [8]. This is supported by the fact that at one specific site where pseudouridylation takes place we find, using an oligonucleotide ligation technique, that there is more U at that site in RNA from mutant cells. There is much less U at the site than in a molar equivalent of RNA synthesized in vitro and therefore containing 100% U. This suggests the mutant dyskerin enzymes convert U to Ψ with less than 100% efficiency, in keeping with the fact that dyskerin mutations are nearly all missense mutations that likely retain function, complete lack of dyskerin being lethal [14]. Indeed in mature steady-state ribosomal RNAs from human cells with pathogenic *DKC1* mutations no difference in the U:Ψ ratio could be detected [7].

Ribosomal RNAs have extensive regions of GC rich hairpins and are consequently notoriously difficult to denature [15]. Our experiments suggest that, using the normal conditions for denaturing RNA for Northern blots, rRNAs are not completely denatured. Since increasing the time of denaturing decreases the mobility of the RNAs, and the mutant RNAs tend to run faster we may conclude that RNAs that lack the full complement of Ψs are more difficult to denature. This is somewhat unexpected since Ψs are thought to stabilize secondary structure. Perhaps the finding of Ψs clustered in functional regions of RNAs [1] favours a particular conformation rather than a more stable one, or adds a degree of flexibility to the secondary structure.

While newly synthesized RNAs show the mobility difference the difference decreases with longer chase times, suggesting rRNA that lacks a full complement of Ψs is relatively unstable. It has been speculated that a defect in ribosome biogenesis may add to the established telomerase defect in dyskeratosis congenita. Our data suggest that rRNA in cells with *DKC1* mutations may have a component that is rapidly turned over, making the cell expend extra energy to overcome this defect and possibly leading to a slowing of growth. Whether this contributes to the DC phenotype remains to be determined.

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